COMPARISON OF FUNGAL COMMUNITIES ASSOCIATED WITH *ORBICELLA FAVEOLATA* IN APPARENTLY HEALTHY AND WHITE PLAGUE-LIKE DISEASED STATES

by

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Comparison of Fungal Communities Associated with *Orbicella faveolata* in Apparently Healthy and White Plague-Like Diseased States

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List of Abbreviations

AB .....alcian blue
AH.....apparently healthy coral tissue from an unaffected colony
AHD.....apparently healthy coral tissue on a diseased colony
BLAST.....Basic Local Alignment Search Tool
BR. .....Bermuda
D.....coral tissue from the tissue-loss margin
DNA.....deoxyribonucleic acid
DSS.....dark spot syndrome
EtBr.....ethidium bromide
FKNMS.....Florida Keys National Marine Sanctuary
H&E.....hematoxylin and eosin
ITS.....internal transcribed spacer
ITS1.....first internal transcribed spacer
LC.....Little Cayman Island
LSI.....Lee Stocking Island, The Bahamas
MBAC.....The MicroBiome Analysis Center
NCBI.....National Center for Biotechnological Information
NMDS.....non-metric multidimensional scaling
One-way ANOVA.....one-way analysis of variance
OTU.....operational taxonomic unit
PAS.....periodic acid-Schiff reagent
PAST.....Paleontological Statistics Software Package for Education
PCR.....polymerase chain reaction
PGM.....Ion Personal Genome Machine®
PERMANOVA.....permutational multivariate analysis of variance
rDNA.....ribosomal deoxyribose nucleic acid
RDP.....Ribosomal Database Project
STX.....St. Croix, U.S. Virgin Islands
WPLD.....white plague-like disease
YBD.....yellow-band disease

Abstract

COMPARISON OF FUNGAL COMMUNITIES ASSOCIATED WITH *ORBICELLA FAVEOLATA* IN APPARENTLY HEALTHY AND WHITE PLAGUE-LIKE DISEASED STATES

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Fungi are a community associated with the coral holobiont, but their participation in and potential impact during a diseased state is inadequately reported. The differences in fungi present in healthy and white plague-like diseased samples of the reef-building Caribbean coral O*rbicella* (formerly *Montastraea*) *faveolata* were explored using preferential staining to identify the location of fungi within the coral coupled with NextGen sequencing to identify specific types of fungi within the community. Distinct fungal communities were linked to coral sampling locations, and in at least one specific location, the Florida Keys National Marine Sanctuary, a correlation between coral health and the associated fungal communities was observed. A more complete understanding of the fungal communities associated with healthy and diseased specimens of *O. faveolata* should allow for better insight into white plague-like diseases of corals and will hopefully

contribute to a more rational approach to reef management.

Introduction

Coral Reefs

Coral reefs provide a multitude of ecosystem services, including food, biotechnological prospects, ecotourism opportunities, and barrier protection from harsh weather (Bourne et al. 2009). Additionally, their structures enhance biodiversity by providing habitats for an assortment of organisms (Bourne et al. 2009). Over the past decades, coral diseases have been increasingly observed (Aronson and Precht 2001; Patterson et al. 2002; Sutherland et al. 2004; Weil et al. 2006; Sunagawa et al. 2009; Fisher et al. 2012; Burge et al. 2013a; Gleason et al. 2017a; Gleason et al. 2017b) and these diseases have led to impairments in the services they provide. (Aronson and Precht 2001; Sutherland et al. 2004; Weil et al. 2006; Bourne et al. 2009; Kimes et al. 2013; Gleason et al. 2017a; Gleason et al. 2017b).

Corals are a broad and diverse group of animals, from the phylum Cnidaria, consisting of two taxonomic classes, Anthozoa and Hydrozoa (Cairns 2007). Within the Class Anthozoa, the Order Scleractinia is notable for the production of calcium carbonate. These corals are often referred to as “stony corals” or “true corals”, and are responsible for creating reefs. Diversity within this order is substantial; Cairns (2007) estimated that 1,482 different species have been identified, including the

species of focus in this study, *Orbicella* (formerly *Montastraea*) *faveolata.*

Like most animals, corals are home to a complex group of microbes that act to provide several functions. The term "holobiont" refers to the entirety of the living communities found in association with the coral and encompasses viruses, bacteria, archaea, protists, fungi, endolithic algae, and zooxanthellae (Rohwer et al. 2002; Dimond and Carrington 2007; Bourne et al. 2009). A remarkable feature of many corals, including tropical Scleractinia, is the incorporation of zooxanthellae in their gastrodermal cells. These symbiotic partners are perhaps the most important community of the holobiont.

Zooxanthellae are a variety of species of symbiotic dinoflagellates. In many coral species, as much as 95% of the energy used by the coral is acquired from its zooxanthellae (Muscatine 1973; Muscatine et al. 1981). Because of this symbiotic relationship, hermatypic corals are able to grow at a much faster rate than ahermatypic corals (Muscatine 1973) and the extra photosynthate provided to the coral cells allows the coral to create new skeleton and secrete mucus (Davies 1984; Falkowski et al. 1984; Crossland 1987; Edmunds and Davies 1989).

The delicate physiological balance the corals must maintain between the zooxanthellae and themselves is essential. During times of stress, such as a temperature change or disease, the coral-zooxanthellae relationship is degraded in a phenomenon known as "bleaching"(Brown 1997; Rosenberg and Ben-Haim 2002; Wegley et al. 2007; Lesser et al. 2007). In a temporary effort to save energy and nutrients, a disruption in the relationship between corals and their photosymbiotic

partners occurs (Rosenberg and Ben- Haim 2002; Wegley et al. 2007). This might provide a short-term solution, allowing the coral to devote all its energy to its own health, but chronic stress will result in a lack of energy and eventual starvation (Rosenberg and Ben-Haim 2002). Twenty to 40% of the photosynthates the corals use for metabolic energy is spent secreting mucus (Davies 1984; Crossland 1987; Edmunds and Davies 1989); and this mucus is, itself, a medium for many other microbes to use as a potential energy source (Brown and Bythell 2005).

When a bleaching event occurs, the mucus levels are impacted, this then causes an interruption in resources for a variety of organisms (Mullen et al. 2004). Many studies have centered on the hypothesis that symbiotic partners switch from being beneficial or benign communities to opportunistic pathogens, attacking or causing damage to the coral (Bentis et al., 2000; Alker et al. 2001; Golubic et al. 2005; Harvell et al. 2007; Lesser et al. 2007; Raghukumar and Ravindran 2012; Yarden 2014). Rather than being primary causes for disease, these shifts in community size and function result in further complications to an already stressed coral (Lesser et al. 2007; Sunagawa et al. 2009). The coral holobiont is suspected to play a role in defense against pathogens and a disruption in this balance can lead to opportunistic infections (Alker et al. 2001; Harvell et al. 2007; Lesser et al. 2007; Golberg et al. 2011; Zhang et al. 2012; Moree et al. 2014; Lehnert et al. 2014).

Orbicella* (formerly *Montastraea*) *faveolata

The primary focus of this study concerns the species, O*rbicella* (formerly

Montastraea) *faveolata,* a stony coral, which is considered to be a critical component of the Caribbean and Western Atlantic reef systems (Knowlton et al. 1992; van Veghel and Bak 1993; Knowlton et al. 1997; Levitan et al. 2004; Gutiérrez-Isaza et al. 2015) **(Figure 1).** At present, the species is listed as endangered by the IUCN (Aronson et al. 2008).

Orbicella faveolata, was once assigned to the genus *Montastraea*, expressly as belonging to the *"M. annularis* species complex" and sub-specifically identified as *M. faveolata.* Three currently recognized species were, for a time, referred to as "*Monastraea annularis* species complex", with specific morphological designations *M. franksi, M. annularis*, and *M. faveolata* that were often used in literature to designate a coral colony that possessed a particular feature (e.g., scattered white-protuberant knobs, separation into upright columns, smooth massive with elevated knobs in rows). Arguments concerning the taxonomic designation varied (van Veghel and Bak 1993; Weil and Knowlton 1994; Veron 1995); but often, abiotic environmental factors and regional variation along a wide range were cited as explanations for the differences observed (Knowlton et al. 1997).

Several studies presented evidence that, what once was thought to be phenotypic variations within a single species, are actually three distinct species, the first being Knowlton et al. (1992) and Weil and Knowlton (1994). And while there is a large degree of overlapping characteristics, evidence in support of this new classification includes the appearance of different colony morphologies in the same location (van Veghel and Bak 1993), differing spawning times (Knowlton et al. 1997;

Levitan et al. 2004; Fukami et al. 2004), oocyte size and number (Levitan et al. 2004), depth preferences (Levitan et al. 2004), varying corallite morphology (Knowlton et al. 1992; van Veghel and Bak 1993), rates of calcium deposition (Knowlton et al. 1992; van Veghel and Bosscher 1995), aggressive behavior toward each other (van Veghel et al. 1996), and partially incompatible gametes (Knowlton et al. 1997; Levitan et al. 2004). Molecular identification among the three species has proved troublesome, but can be achieved by analyzing multiple loci (Fukami et al. 2004). Although recognized as distinct species, and reclassified into a different genus, both past and present studies concerning these animals may refer to them using their former name (Aronson et al. 2008).

Orbicella franksi and *O. annularis* can be differentiated from *O. faveolata* by their denser skeletons and wider range of depth(Knowlton et al. 1992; van Veghel and Bosscher 1995). *Orbicella faveolata* is commonly referred to as mountainous star coral, aptly named due to the mountain-like bumps that colonies form on otherwise flat surfaces and the star-shaped structure characterizing the corallite outline. Among the three members of the genus, *O. faveolata* is easiest to identify with molecular studies and seems to have diverged from the other members earlier in its evolutionary history (Fukami et al. 2004)

Figure 1. Map demonstrating the geographic range of *O. faveolata*. Courtesy of IUCN data provided by Aronson et al. (2008).

Complexities of Coral Diseases

A growing consensus centers on the notion that coral diseases are the result of a disruption between host, agent (pathogen), and environment (Lesser et al. 2007; Bourne et al. 2009; Yarden 2014; Meyer et al. 2014). Changes in any one of these factors influences the other two points of the "disease triangle" and offers distinctive scenarios suggesting specific investigations (Bourne et al. 2009). An increasingly supported perception is that coral diseases come from environmental stressors allowing opportunistic infections (Lesser et al. 2007; Muller and van Woesik 2012; Meyer et al. 2014; Yarden 2014; Gleason et al. 2017a; Gleason et al. 2017b). Coral diseases are becoming more prevalent and causing more severe damage, resulting in a dramatic decrease in abundance and diversity of coral species (Brown 1997; Patterson et al. 2002; Hoegh-Guldberg et al. 2007; Littman et al. 2011; Soffer et al. 2014; Gleason et al. 2017a; Gleason et al. 2017b).

Coral diseases are often named, described, and characterized by their physical appearances (Roder et al. 2014). This trend has proved problematic in that different diseases can often manifest themselves with very similar signs (Denner et al. 2003; Thompson et al. 2006; Work et al. 2012; Kellogg et al. 2013; Roder et al. 2014), and known pathogens can induce different responses in different coral species (Weil 2004; Renegar et al. 2008; Sunagawa et al. 2009; Work et al. 2012; Kellogg et al. 2013; Roder et al. 2014; Work and Meteyer 2014). Bruckner notes that thirteen different "white diseases" have been suggested; each with varying degrees of host susceptibility (2016). This category of diseases includes tissue loss that does

not seem to be related to predation nor caused by a pigmented microbial mat, which results in the appearance of the bare white skeleton that is normally covered by brownish coenenchyme tissue and polyps.

For example, Denner et al. (2003) identified *Aurantimonas coralicida* as the bacterium responsible for white plague II in the Caribbean; while Thompson et al. (2006) demonstrated that *Thalassomonas loyana* was the bacterium responsible for a white plague-like disease in the Red Sea. Furthermore, in another study of the Caribbean Sea, Sunagawa et al. did not report the presence of *A. coralicida* in either healthy or diseased samples of *O. faveolata* (2009).

This type of situation is not exclusive to bacteria either. One identified fungal pathogen of coral is *Aspergillus sydowii*, whose presence has complicated the interpretation of the disease state in the sea fan genus *Gorgonia* (Nagelkerken et al.1997; Smith et al. 1996; Geiser et al. 1998; Kim and Harvell 2004; Burge et al. 2013b; Yarden 2014). Interestingly, Barrero-Canosa et al. (2013) found multiple opportunistic parasitic fungi in sea fans affected by aspergillosis. The presence of numerous other fungi within the sea fans led many authors to speculate that different microorganisms could be involved in the disease (Barrero-Canosa et al. 2013; Yarden 2014; Gleason et al. 2017a). Furthermore, healthy sea fans have been shown to house the pathogen *Aspergillus sydowii (*Soler-Hurtado et al. 2016*)* which was previously reported as being responsible for an outbreak in the same types of soft corals, *Gorgonia ventalina* and *G. flabellum* (Nagelkerken et al. 1997; Smith et al. 1996; Geiser et al. 1998; Kim and Harvell 2004). Conversely, the same pathogen is

not always present in investigations of sea fans exhibiting signs of this disease (Toledo-Hernández et al. 2008). Partially explained as opportunistic pathogens (Barrero-Canosa et al. 2013; Yarden 2014; Gleason et al. 2017a; Gleason et al. 2017b), there remain unidentified environmental factors that may play a role in disease.

Unfortunately, the inability to clearly distinguish one disease from another, or link macroscopic signs to specific microbial pathogens makes it difficult to develop management strategies (Sunagawa et al. 2009; Kellogg et al. 2013). The same disease could display different signs during different stages of progression (Richardson 1998) and therefore be misidentified as a different type of disease. The same disease has been known to produce different signs in different species. Different signs (lesions) can also appear on different parts of the polyp and in different locations of a coral colony. All of these difficulties are further complicated by the environment within which corals live (Work and Meteyer 2014) and their study requires multiple techniques and expensive equipment.

White Plague

Of particular significance are coral diseases showing signs of white plague, an epidemic that was first documented in the Caribbean by Dustan (1977) and subsequently Richardson et al. (1998a)reported a similar, but differently identified disease that affected many observed species of coral within the same area. Since then, at least thirteen different types of "white syndromes" have been proposed

(reviewed in Bruckner 2016).

A defining feature of white plague is persistent loss of tissue (Richardson et al. 1998a; Richardson et al. 1998b; Richardson et al. 2001; Denner et al. 2003; Pratte and Richardson 2016). As the disease spreads, the boundary of healthy tissue recedes and the coral's skeleton is exposed, taking on the appearance of a growing white zone (Richardson et al. 1998a; Richardson et al. 1998b; Denner et al. 2003; Pratte and Richardson 2016). White plague was originally classified into three types (I, II and III), distinguished by rate of tissue loss (Soffer et al. 2014), species affected, and prevalence (Richardson et al. 1998a, Richardson et al. 1998b; Richardson et al. 2001; Denner et al. 2003; Pratte and Richardson 2016). A developing trend now, due to the similarities among the different types, is to collectively refer to lesions as "tissue loss"(Work and Aeby 2006). In more recent literature, outbreaks of white diseases are sometimes distinguished by location. Caribbean cases of acroporid corals exhibiting gross signs of tissue loss are sometimes designated as "white band" and "white pox" depending on the species affected and pattern of tissue loss (Bythell et al. 2004) whereas tissue-loss diseases of Indonesia and Southeast Asia are termed "white syndromes"(Bourne et al. 2015).

Generally, white plague-like diseases (WPLD) are distinguished by the manifestation of lesions on a particular coral colony and subsequent spread to new colonies (Pantos et al. 2003; Denner et al. 2003; Soffer et al. 2014). Other coral diseases are often characterized by a pigmented band (such as yellow-band disease or pink-line syndrome), or microbial mat (black-band disease) separating healthy

tissue from lesions. White plague-like diseases lack a distinctive pigmented margin, displaying freshly exposed skeleton sharply contrasted with apparently healthy tissue (Richardson et al. 1998a; Richardson et al. 1998b; Richardson et al. 2001; Denner et al. 2003; Pratte and Richardson 2016; Bruckner 2016). The result of this disease is ultimately the loss of tissue and potential colony death (Richardson et al. 2001; Miller et al. 2006; Wei et al. 2006).

Disease transmission can be extremely fast; several millimeters or even centimeters of linear tissue loss can occur daily (Richardson 1998; Richardson et al. 1998b; Pantos et al. 2003; Soffer et al. 2014). Two studies have presented evidence that bacterial infections might be responsible such as *Aurantimonas coralicida* in the Caribbean (Denner et al. 2003) and *Thalassomonas loyana* in the Red Sea (Thompson et al. 2006). But neither microbe is reliably found in all disease cases and the cause or causes of WPLD remain ambiguous (Bruckner 2016).

Marine and Endolithic Fungi

Fungi have long been known to exist in the marine environment (Murray 1893; Raghukumar and Ravindran 2012; Yarden 2014; Gleason et al. 2017a; Gleason et al. 2017b) and their association with corals has been recognized for decades (Kendrick et al. 1982). Yarden (2014) reports more than 800 described species of fungi from the marine environment and notes that inevitably, many have yet to be discovered. New reports have found fungi in extreme marine environments such as deep-sea sediments (Schumann et al. 2004; Damare et al. 2006; Bengtson et

al. 2014), methane hydrates (Lai et al. 2007), associated with coral in aphotic zones (Freiwald et al. 1997), oxygen deficient regions (Jebaraj et al. 2010), and in hydrothermal vents (Le Calvez et al. 2009). While fungi are pervasive in marine habitats, their role is insufficiently understood in many coral ecosystems (Golubic et al. 2005; Amend et al. 2012; Yarden 2014).

Marine fungi are broadly lumped into one of two classes. Those originating in the ocean are referred to as "obligate marine fungi." Conversely, "facultative marine fungi" denotes those fungi that have originated in a freshwater or terrestrial environment, but are capable of survival and reproduction in the ocean. Predictably, many of the fungi found in association with coral reefs have their origin on land (or were first described there) and more notably many of those same fungi have been shown to be pathogenic elsewhere (Kim and Rypien 2016). Fungi can be found on the surface or in oral cavities of reef organisms as well as in the calcium carbonate substratum formed by coral skeletons. The latter endolithic fungi can be true burrowers "euendoliths" or "cryptoendoliths," organisms that take advantage of previously created tunnels (Priess et al. 2000; Golubic et al. 2005; Gleason et al. 2017a).

Fungi are a life-long part of a coral's growth and survival. According to Le Campion-Alsumard et al. (1995b), endolithic fungi colonize coral larvae within hours so they can begin to bore through their skeletons as soon as they are produced. Raghukumar and Ravindran (2012) even proposed the possibility of vertical transmission, citing evidence from sponges. Maldonado et al. (2005)

showed that chitin-walled yeast cells were actually present in fertilized eggs of three species of sponges from the genus *Chondrilla*. This evidence led Raghukumar and Ravindran (2012) to speculate that fungal cells might also inhabit coral polyp coelenterons (gastrovascular cavities) allowing them access to coral gametes before spawning.

Along with the possibility of residence within the gastric cavity, fungi are known to occupy the surface mucopolysaccharide layer (Harel et al. 2008; Damare et al. 2012; Gleason et al. 2017a; Barathikannan et al. 2017) as well as the aragonite exoskeleton (reviewed in Raghukumar and Ravindran 2012; Yarden 2014; Gleason et al. 2017a; Gleason et al. 2017b). In these locations, they can take advantage of nearby bacteria and mucus on the surface, and algae, microeukaryotes, and coral secretions as nutritional resources from below. Fungi are also believed to be parasitic or opportunistic pathogens, at times exploiting the coral host (Le Campion-Alsumard et al. 1995a; Kendrick et al. 1982; Priess et al. 2000; Brown and Bythell 2005; Golubic et al. 2005; Yarden 2014).

Several authors have demonstrated immune or defensive responses in corals toward fungi, indicating their parasitic role (Le Campion-Alsumard et al. 1995b; Bentis et al. 2000; Domart-Coulon et al. 2004; Golubic et al. 2005; Raghukumar and Ravindran 2012; Yarden 2014; Moree et al. 2014). Supporting the pathogenic hypothesis is the observation that the fungi residing within a coral's skeleton have been shown to burrow upward at a rate that matches the host's calcium carbonate deposits (Lukas 1974; Le Campion- Alsumard et al. 1995a). This corresponding pace

indicates that the coral's calcium deposits are deliberate so that the basal body wall avoids direct interaction with the fungi (Golubic et al. 2005). Additionally, fungi infiltrating into coral tissue can induce modified calcium deposition to avoid further contact (Le Campion- Alsumard et al. 1995a; Domart-Coulon et al. 2004).

Beyond the potential for infection, fungi do provide services within the holobiont. Wegley et al. (2007) performed a metagenomic analysis of *Porites astreoides* and found transcriptional evidence that the fungal community was a key component in recycling nitrogen and thus acts in a mutualistic manner with the host. Kimes et al. (2010) looked at the functional genes present within the holobiont of *Orbicella (Montastraea) faveolata* which showed that coral-associated fungi were connected with polysaccharide degradation, metal homeostasis, nitrogen cycling, and "xenobiotic contaminant degradation" . Domart-Coulon et al. (2004) selectively isolated a strain of *Cryptococcus* from laboratory-maintained colonies of the coral *Pocillopora damicornis* and found that the cultured coral cells exposed to the fungus remained viable for up to two days longer than cells cultured without the fungus. Cristianawati et al. (2017) isolated a stain of *Trichoderma* from the coral *Favia* sp. capable of inhibiting growth of multidrug-resistant *Staphylococcus haemolyticus.* As more fungal-based and metagenomic studies on coral are conducted, our understanding of their contributions to healthy coral will be further enhanced (Golubic et al. 2005).

Histology

To determine not only the cause of disease, but also where the disease is taking place and the host's immune response, microscopy is essential. Histological examination of coral tissues allows for the visualization of pathogens, coral necropsy, and lesions as well as the coral's immune response (Work et al. 2012; Work and Meteyer 2014). Many molecular ecology and culture-based studies on coral failed to provide histological evidence and in failing to do so provided inadequate or incomplete constructs of the disease (Work and Meteyer 2014) which, in some cases may have resulted in future research being misguided to incorrect or unnecessary areas (Work et al. 2008; Work et al. 2012 Work and Meteyer 2014). In a survey of coral disease papers published between 1965 and 2013, Work and Meteyer (2014) found that only 12% of those studies incorporated light microscopy. Noting the seriousness of this, the authors state, "without the histology component of the data that provides insight into cell pathology and host response at the microscopic level, documentation of the relationship between the host, agent, and environment is incomplete" (Work and Meteyer 2014). Molecular-based studies can detect distinctive microbial communities within coral having a specific impairment but are not capable of demonstrating if those microbes are responsible for the tissue damage and macroscopic signs of disease (Work and Meteyer 2014).

For this study, histopathology provides a way to determine where the fungi can be found in association with the coral. Additionally, histopathology allows for

the visualization of the corals' immune response if occurring. Finally, because of the overlapping ranges and similar features within the members of the genus *Orbicella*, histology also allows for correct species confirmation. *Orbicella faveolata* houses specific internal cells not found in *O. franksi* or *O. annularis* (personal communication with E. Peters) and the presence of these cells confirms that the correct species was selected for sampling.

Hematoxylin and eosin (H&E) staining is a standard procedure for identifying the integrity of the coral polyp. In overwhelming infections, fungi can be identified using H&E stains, but in cases of mild infection, or even in recovery, fungal diagnosis is difficult (Peabody et al. 1955). Typically, a hematoxylin and eosin (H&E) stain is done alongside a preferential stain, such as the periodic acid-Schiff reagent (PAS) procedure, which is an accurate method for identifying fungi. Alcian blue, PAS, and hematoxylin (AB/PAS/H) staining can be used to differentially stain between neutral and acidic mucopolysaccharides. PAS is an established stain for histological diagnoses of fungi (Peabody et al. 1955; Carson and Hladik 2009) and takes advantage of the carbohydrate-rich fungal cell wall. Carbohydrates, neutral mucopolysaccharides, and mucoproteins are oxidized with periodic acid to form aldehydes. Schiff reagent then combines with those newly formed aldehydes to produce a purple or magenta coloration depending on the counterstain used (Guarner and Brandt 2011). Several other staining procedures can help to identify fungi, most notably Gomori methenamine silver (GMS). However, in the case of coral, PAS stains are preferred due to the enhanced intracellular detail that can be

obtained and viewed within the fungi (Kradin and Iafrate 2010).

Culture-Independent Studies

Diagnostic methods using molecular techniques to assess the microbial profile of a sample are becoming increasingly critical to the diagnosis of marine organisms. As much as 99% of marine microorganisms cannot be cultured in a laboratory setting (Amann et al. 1995; Fuhrman and Campbell 1998; Pantos et al. 2003; van Dorst et al. 2014). Despite this, few coral studies have utilized culture-independent approaches on fungi (Raghukumar and Ravindran 2012). Several papers have reported the presence of fungi within the coral skeleton but had deficiencies in properly identifying the fungal taxa (Kendrick et al. 1982; Bak andLaane 1987; Le Campion-Alsumard et al. 1995a; Le Campion-Alsumard et al. 1995b; Bentis et al. 2000; Priess et al. 2000; Morrisson-Gardiner 2002, Gutie´rrez-Isaza et al. 2015).

Phylogenetic and community identification studies often rely on sequencing a portion of transcribed DNA corresponding to ribosomal RNA (rRNA). For example, bacterial and archaeal communities are assessed by sequencing a region of the 16s rRNA gene. Several sequence databases hold previously sequenced and mapped genomes, along with phylogenetic information for comparison. By comparing newly sequenced samples with those published and available through sequence databases, microbial communities can be identified and analyzed for diagnosis.

Fungal genomes consist of hundreds of repetitive ribosomal gene clusters

(Moore et al. 2011). “The areas that lie between the 18S and 5.8S and between the 5.8S and 28S genes are called internally transcribed spacers (ITS1 and ITS2) (Moore et al. 2011).” Differing portions of a fungus’ ribosomal gene cluster, including sequences separating different rRNA genes, can be used to identify fungi with varying degrees of specificity (Moore et al. 2011). This study examined the ITS1 region, a portion of the operon that is helpful in distinguishing between different “species of fungus and closely related genera” (Moore et al. 2011).

Objectives

The contributions coral reefs provide to ecological health cannot be overstated and it is critical to do as much as possible to preserve these ecosystems. As such, it is imperative that diseases, such as those that manifest with white plague-like signs, are fully investigated to understand disease etiology. The purpose of this study, encompassed four primary goals, focusing on the coral species *Orbicella* (*Montastraea) faveolata* and their associated fungal community, to:

(1) determine whether the fungal communities within *O. faveolata* vary temporally, spatially, or in any other noticeable manner;

(2) demonstrate the presence of fungi using histopathological techniques and record any abnormalities or noticeable tissue infiltration;

(3) characterize the fungal community profile of *O. faveolata* in samples perceived as healthy and those identified with signs of white plague like disease; and

(4) identify any potential indicator species within the fungal community that are consistently associated with diseased corals of *O. faveolata*, but absent in apparently healthy samples.

Several species-specific studies have shown fluctuations in the bacterial communities of apparently healthy and diseased corals. Interactions between other members of the holobiont and the host coral are likely to shift during the period of progression when disease occurs. As Bourne et al. (2009) noted, "The dearth of knowledge about the actual causes and pathogenesis for many of these diseases is largely the result of too few researchers studying the interactions between the

putative causative agents and the coral host, limitations in funding, and a complex host for which a basic understanding of physiology is still lacking." Characterizing the fungal profile of *Montastraea (Orbicella) faveolata* will assist in addressing this shortcoming.

Hypotheses

$H1_0$: Samples of *Orbicella faveolata* exhibit no discernable variation in endolithic fungal communities by location.

$H1_A$: Samples of *Orbicella faveolata* harvested from the same location exhibit similar fungal community profiles compared to those from different locations.

$H2_0$: Samples of *Orbicella faveolata* display no endolithic fungal community variations between apparently healthy coral samples and those exhibiting signs of white plague-like disease.

$H2_A$: Greater fungal richness occurs in samples of *Orbicella faveolata* exhibiting signs of white plague-like disease.

$H3_0$: Specific fungal species are not associated with samples of *Orbicella faveolata* exhibiting signs of white plague-like disease.

$H3_A$: Fungi are specifically associated with samples of *Orbicella faveolata* exhibiting signs of white plague-like disease.

Methods

Samples

Previously collected samples by Cook (2009) **(Figure 2)** of *Orbicella (Montastraea) faveolata* skeleton and tissue, exhibiting apparently healthy and white plague-like signs, were used for this analysis. Samples were designated into one of three classes: apparently healthy tissue taken from an apparently healthy colony (AH), apparently healthy tissue taken from a diseased colony (AHD), and diseased tissue taken from the tissue-loss margin of a colony exhibiting signs of white plague(D). In total, samples from five different locations (Florida Keys, Cayman Islands, Bahamas, St. Croix, and Bermuda) were used for analysis. These samples had been fixed, enrobed in 1.5% agarose to trap material on or in the skeleton, then decalcified in 10% ethylenediaminetetraacetic acid at pH 7, and embedded in paraffin wax (Peters et al. 2005; Cook 2009). Additional samples had been previously crushed, ground, the DNA extracted, and stored at -80 °C for molecular analysis.

Staining

Embedded samples, collected by Cook (2009) were sectioned at a 5-µm thickness using an Olympus CUT 4060 microtome (Olympus America Inc., Center Valley, PA),

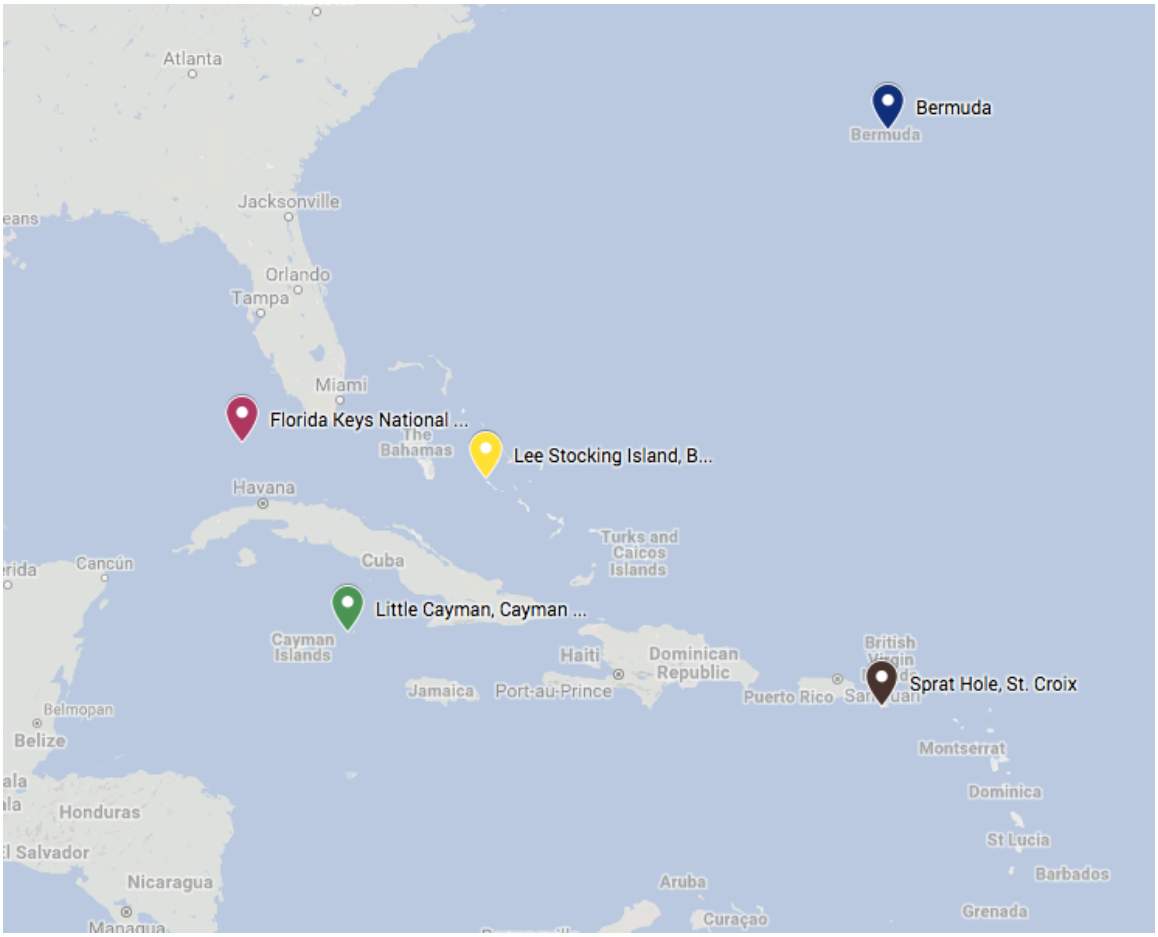

Figure 2. Map illustrating the sampling locations from Cook (2009). Samples from the Florida Keys, Cayman Islands, Bahamas, St. Croix, and Bermuda (designated on map) were also used for the fungal analysis in this study. Generated using Google Maps.

and the resulting ribbons were floated in a 45 °C bath. Twin sections from each embedded sample were then mounted on microscope slides for staining. The sections were stained with H&E or AB/PAS/H, respectively, according to the methods outlined in Carson and Hladik (2009) and written as George Mason University Histology Laboratory standard operating procedures. The samples were then coverslipped with Permount™ mounting medium (Thermo Fisher Scientific, Inc., Waltham, MA). Resulting slides were examined for coral structural appearance and the occurrence of fungi using light microscopy.

DNA Amplification and Sequencing

Fungal-specific primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS2 FAM (**5'-** GCTGCGTTCTTCATCGATGC**-3'**) (White et al. 1990) were used to amplify the Internal Transcribed Spacer Region 1 (ITS1) from the DNA extracted from the coral samples by Cook (2009). A PCR master mix containing 7.9 µl of DEPC water, 2 µl of 10X PCR Rx. buffer for Taq Gold polymerase, 2 µl of 25 mM Mg mix, 2 µl of 2 mM each (dNTPs) deoxynucleotide triphosphate, 1 µl (10 µM) of both the forward and reverse primer, 2 µl 0.1% BSA, and 0.1 µl Taq Gold polymerase (5 units/µl) per sample was added to a 1.5 mL Eppendorf tube. Master mix (18 µl) was aliquoted into 0.2-mL PCR tubes along with 2 µl of extracted DNA from each sample. DNA amplification was performed in a 20 µl final volume solution per reaction.

Polymerase chain reaction was done using an Applied Biosystems® GeneAmp® PCR System 9700 thermocycler (Life Technologies, Frederick, MD). DNA was initially denatured at 95 °C for 11 minutes. Subsequently, 35 cycles consisting of denaturation at 95 °C for 30 seconds, primer annealing at 50 °C for 30 seconds, and primer extension at 72 °C for 2 minutes +5 seconds per cycle were conducted (White et al. 1990). A final extension was implemented at 72 °C lasting 30 minutes (White et al. 1990). Reactions were then cooled and stored at 4 °C. DNA products were visualized on a 1% agarose gel in TAE buffer with EtBr for confirmation. Samples appearing to have low band strength were reamplified using 2 µl of product from the initial reaction.

In total, 84 coral samples were analyzed using the Ion Torrent™ platform to determine the associated fungal communities within each sample. Among the 84 samples, 73 samples belonged to the species *O. faveolata* and 11 samples to *O. franksi.* Sequencing by synthesis was performed according to the methods of Sikaroodi and Gillevet (2012) using the forward ITS1 primer, (5'TCCGTAGGTGAACCTGCGG-3') (White et al. 1990) with sample specific barcode sequences and the aforementioned ITS2 FAM reverse primer (White et al. 1990).

Statistical Analysis

All generated sequences were subjected to a nucleotide BLAST® search through George Mason University's MBAC Galaxy Portal. Following taxa assignment, all non-fungal identities were eliminated from the FASTA file before further

analysis. Once all undesired sequences were deleted, the remaining 130,177 fungal sequences were identified using the RDP 11 database (Wang et al. 2007; Cole et al. 2014) using the following parameters to identify taxa: bootstrap greater than 0.70 designated as "OTHER." bootstrap less than 0.70 and greater than 0.10 designated "UNKNOWN," and a bootstrap less than 0.10 to annotate genera.

The identified fungi within each sample were uploaded into Microsoft Excel. Classifications specifically designating anamorph (morphology of the asexual stage) and corresponding teleomorph (morphology of the sexual stage) assignments were combined; further analyses were done assessing them as a single taxon. Each sample's fungal profile was normalized to reflect the composition of fungal species (OTUs) identified through sequencing as expressed by their percent of the total community (relative abundance). Fungal presence in individual coral samples was used to create histograms using the chart/stacked column option in Microsoft Excel (Gillevet et al. 2009). Individual profiles also were averaged by sample collection location, with resulting averages used to generate pie charts showing most common types of fungi found in a location.

Coral samples were examined according to normalized fungal taxa (OTU sequences). Samples were classified according to coral condition and then separately according to location. One-way analysis of variance (one-way ANOVA) and non-metric multidimensional scaling (NMDS) statistical evaluations were carried out using PAST statistical software (Hammer et al. 2001). All program default settings were used. A Euclidean similarity index measure was used to

generate a two-dimensional scatter plot. A distance-based one-way PERMANOVA was then run to make pairwise comparison between the ordination configurations produced by NMDS. Default settings were also used for PERMANOVA analysis and included Euclidean similarity index and 9999 permutations. The resulting output was compared using a 0.05 p-value as a cut off for statistical significance. Indicator species analysis was conducted using the statistical software PC-ORD (McCune and Mefford. 2011). Indicator values were calculated with default settings according to Dufrêne and Legendre (1997) using a 0.05 p-value as a cut off for statistical significance.

Results

Thirteen of the 22 preferentially stained (AB/PAS/H) slides of the samples demonstrated conclusive evidence of fungal inhabitation. No fungi were observed in the polyps' mouths or epidermis of coral tissues. Endolithic fungal infiltration into coral tissue was measured twice. An H&E stained sample of *O. franksi* (prepared by Cook 2009) showed evidence of tissue infiltration **(Figure 3).** Furthermore, an AB/PAS/H stain revealed that a sample of *O. faveolata* appeared to show tissue infiltration by fungal hyphae **(Figure 4)**. No fungal reproductive structures could be identified in any of the histologic preparations.

A total of 130,177 fungal reads were obtained from the 84 samples. Eleven samples of *O. franksi* all came from one location (Bermuda) and produced 5393 reads, with three fungal taxa exclusively found in *O. franksi*. Three additional fungal taxa were assigned to the Basidiomycota. Seventy-three samples of *O. faveolata,* yielding 124,784 reads, were harvested from four sites: Lee Stocking Island in the Bahamas (LSI), St. Croix (STX), Florida Keys National Marine Sanctuary (FKNMS) and Little Cayman Island (LC). The average number of fungal reads for the samples from this species was 1,709. Twenty-four of the initial 84, or 28.6% of samples, were excluded from analysis due to low read count (<50 reads). The remaining 60 fungal

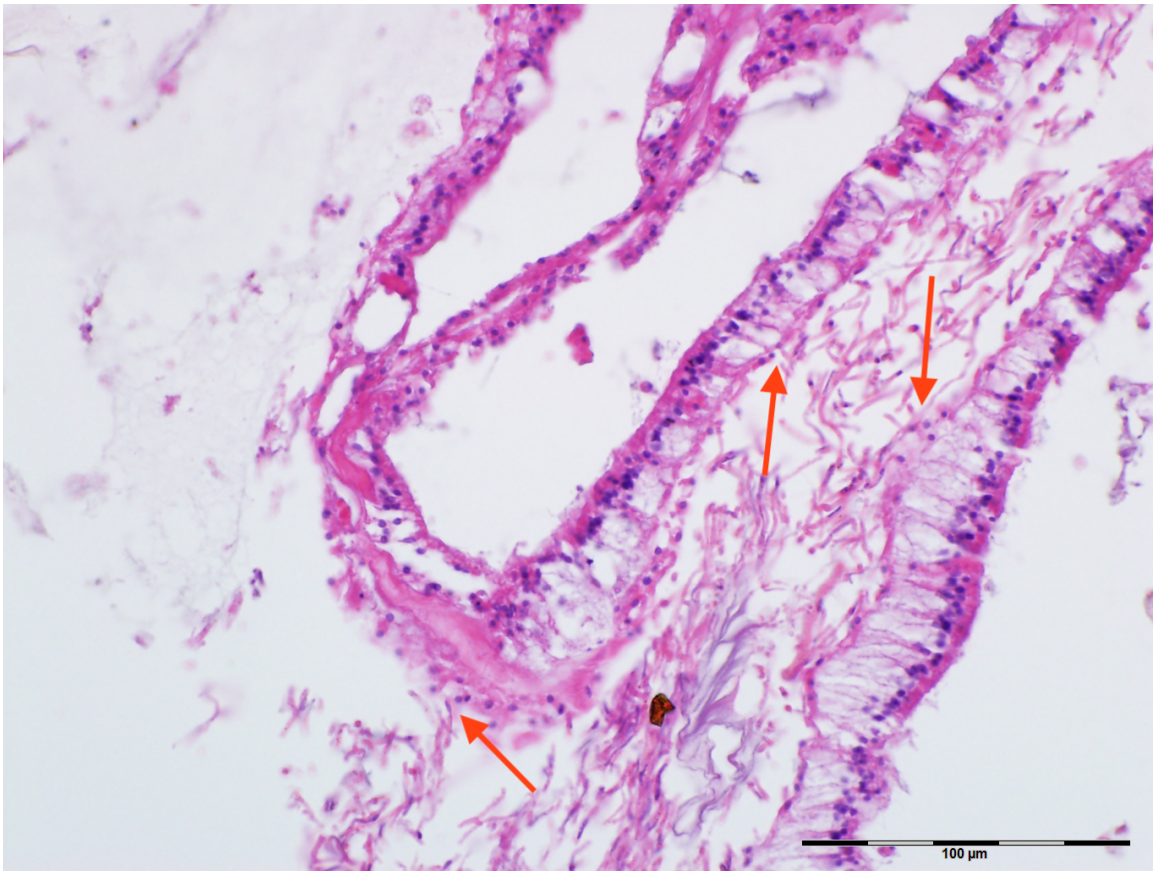

Figure 3. H&E 400x magnification, Sample of *O. franksi* taken from Bermuda that displayed macroscopic signs of "white-plague like" disease. Light microscopy image of what appears to be fungal infiltration (arrows) into coral tissue. In certain cases of infection, identification of fungi can be achieved using morphology without preferential staining. Slide prepared by Cook (2009).

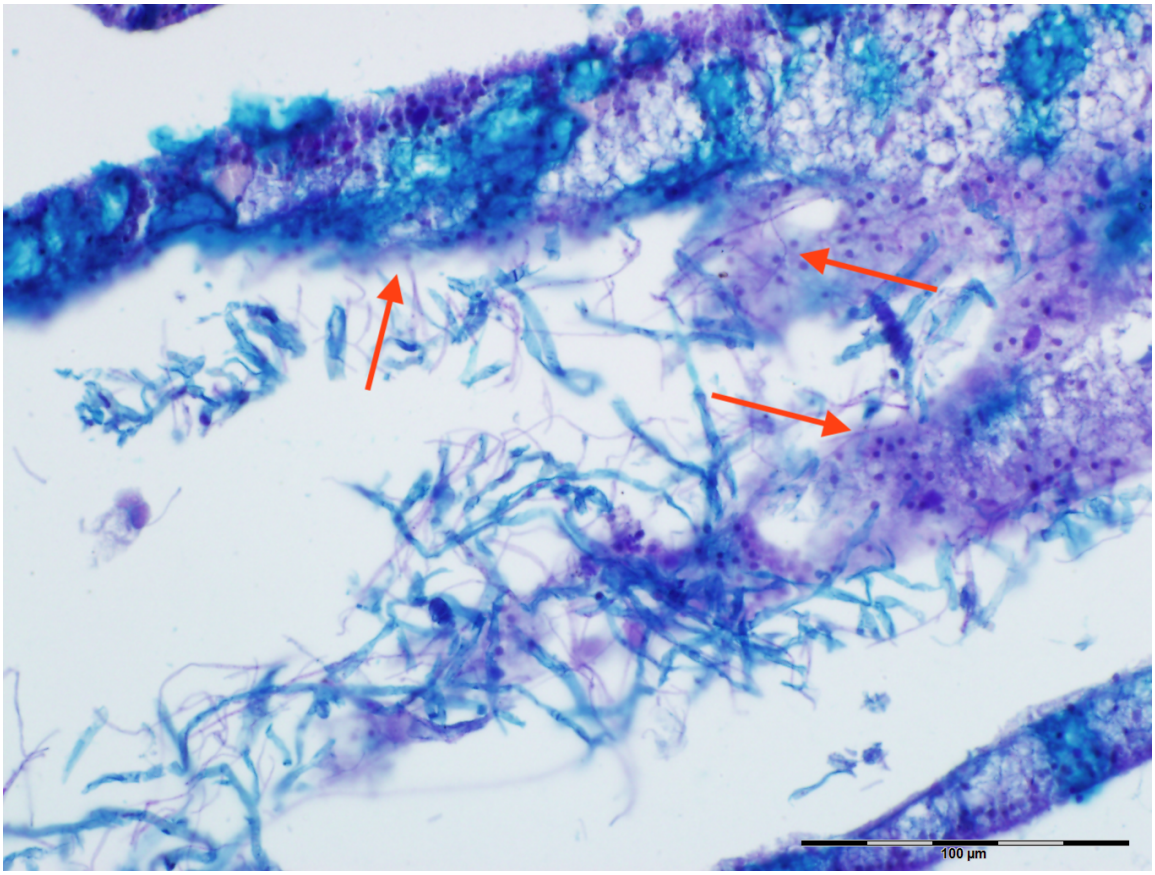

Figure 4. AB/PAS/H 400x magnification, preferential staining showing that there is fungal infiltration into coral tissue calicodermis (red arrows).

profiles (71.4%) were used for histogram construction, and NMDS, and pairwise comparisons.

Among these 60 samples, the average richness was 8.6 taxa per sample. Apparently healthy samples averaged 9.5 taxa, apparently healthy-on-diseased samples averaged 8.0 taxa, and diseased samples averaged 8.2 taxa **(Table 1).** Total richness of the 60 samples yielded 108 distinct taxa that when sequenced were assigned to 3 phyla, 14 classes, 34 orders, 51 families, and 70 different genera. Taxonomic richness was compared among apparently healthy, apparently healthy-on- diseased, and diseased samples among all locations **(Figure 5)**. Specifically, the *O. franksi* samples from Bermuda were dominated by *Arthoderma* with 49% and *Mycena* at 30% of the community profiles, respectively. Other notable taxa included *Mycotypha* (7%) and *Farsizyma* (7%). The Florida Keys samples were dominated by *Arthoderma* constituting 70% of the community profile, followed by *Mycena* with 6% and *Mycotypha* with 4%. The Little Cayman samples were dominated by *Arthoderma* with 58% of the total profile followed by *Mycena* and *Tuber* at 8% each. The Bahamian samples were dominated by *Beauveria/Cordyceps* with 83% of the total profile followed by *Arthoderma* at 15%. The St. Croix samples were dominated by *Arthoderma* with 77% of the total profile followed by *Mycena* at 6%. Many of the taxa identified through sequencing in this study have been previously

Table 1 Average fungal taxa richness of *O. faveolata* and *O. franksi* samples. Anamorphs and corresponding teleomorphs were considered one taxon.

State of Health	Average Richness
Apparently Healthy	9.5
Apparently Healthy on Diseased	8.0
Diseased	8.2
All samples	8.6

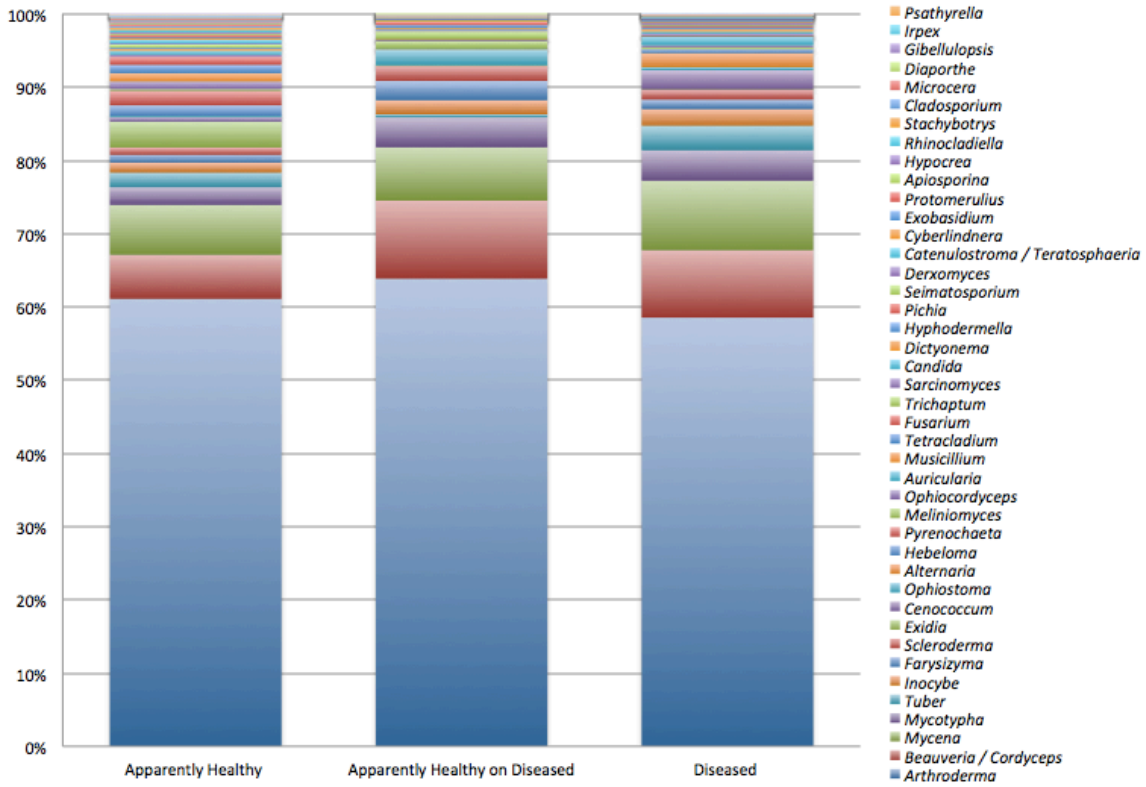

Figure 5. Histogram representing the average community taxa profile of all samples grouped according to state of health. Genera are listed in order of their appearance in bars (from top to bottom).

described in other coral studies **(Table 2).** The results of a one-way ANOVA determined that there were no significant differences among the three health classes based off of fungal community species richness (F=0.72) (d.f.=2) (p =0.49).

When taxa were analyzed based on location, the resulting histograms revealed that *Arthroderma* was the dominant genus at most locations **(Figure 6)**. Non-metric multidimensional scaling (NMDS) was applied to all fungal communities, grouping samples by state of health or location. Regarding state of health, the ordination suggests a large degree of overlap among the disease classes **(Figure 7)**. Among all samples, pairwise comparisons from the one-way PERMANOVA revealed that there were no statistical differences between the fungal communities based on disease state **(Table 3)**.

Ordination and pairwise comparisons did demonstrate that several locations had fungal profiles that were distinctive (p-value <0.05) from others **(Figure 8 and Table 4)**. In summary, samples taken from Bermuda (BR) were distinct from all other locations, except for Little Cayman Island (LC). Samples taken from the Florida Keys National Marine Sanctuary were statistically distinct from the Bermuda site and Lee Stocking Island. Little Cayman Island samples were distinct from Lee Stocking Island and St. Croix. Lee Stocking Island samples were distinct from all other sampling locations. St. Croix samples were distinct from all sampling sites except for the Florida Keys National Marine Sanctuary.

Table 2. All fungal taxa found through RDP11 analysis in this study.

Phylum	Genus	Host	Location	Reference
Ascomycota	*Acremonium*	*Porites lutea*	Arabian Sea	Ravindran et al. 2001
Ascomycota	*Acremonium*	*Pacifigorgia* and other octocorals	Columbia	Barrero-Canosa et al. (2013)
Ascomycota	*Acremonium*	Sponges	Singapore	Koh et al. (2000)
Ascomycota	*Acremonium*	*Meandrina meandrites*	Barbados	Kendrick et al. 1982
Ascomycota	*Acremonium*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Alternaria*	*Acropora formosa*	Great Barrier Reef	Yarden et al. 2007
Ascomycota	*Alternaria*	*Leptogorgia* sp. *and L. obscura*	Ecuador	Soler-Hurtado et al. 2016
Ascomycota	*Alternaria*	Not specified	Great Barrier Reef	Morrison-Gardiner 2002
Ascomycota	*Alternaria*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Apiosporina*	Not reported		
Ascomycota	*Arthroderma*	Not reported		
Ascomycota	*Beauveria*	Not specified	Great Barrier Reef	Morrison-Gardiner 2002
Ascomycota	*Cadophora*	Algae	Not specified	Almeida et al. (2010)
Ascomycota	*Candelaria*	Not reported		
Ascomycota	*Candida*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Capronia*	Not reported		
Ascomycota	*Catenulostroma*	Not reported		
Ascomycota	*Cenococcum*	Not reported		

Phylum	Genus	Host	Location	Reference
Ascomycota	*Cercospora*	Not reported		
Ascomycota	*Cladophialophora*	Not reported		
Ascomycota	*Cladosporium*	*Leptogorgia* sp. *and L. obscura*	Ecuador	Soler-Hurtado et al. 2016
Ascomycota	*Cladosporium*	*Porites lutea*	Arabian Sea	Ravindran et al. 2001
Ascomycota	*Cladosporium*	*Acropora formosa*	Great Barrier Reef	Yarden et al. 2007
Ascomycota	*Cladosporium*	*Montastraea annularis*	Barbados	Kendrick et al. 1982
Ascomycota	*Cladosporium*	*Porites* sp.	Rarotonga	Kendrick et al. 1982
Ascomycota	*Cladosporium*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2007 8?
Ascomycota	*Cladosporium*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Cladosporium*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Cordyceps*	Not reported		
Ascomycota	*Cortinarius*	Not reported		
Ascomycota	*Cosmospora*	Not reported		
Ascomycota	*Cosmospora*	Not reported		
Ascomycota	*Cyberlindnera*	Not reported		
Ascomycota	*Cyphellophora*	Not reported		
Ascomycota	*Diaporthe*	*Pacifigorgia* and other octocorals	Columbia	Barrero-Canosa et al. (2013)
Ascomycota	*Fimetariella*	Not reported		
Ascomycota	*Flavoparmelia*	Not reported		
Ascomycota	*Fusarium*	*Porites lutea*	Arabian Sea	Ravindran et al. 2001
Ascomycota	*Fusarium*	*Leptogorgia* sp. *and L. obscura*	Ecuador	Soler-Hurtado et al. 2016

Phylum	**Genus**	**Host**	**Location**	**Reference**
Ascomycota	*Fusarium*	Sponges	Singapore	Koh et al. (2000)
Ascomycota	*Fusarium*	*Acropora formosa*	Great Barrier Reef	Yarden et al. 2007
Ascomycota	*Fusarium*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Gibellulopsis*	Not reported		
Ascomycota	*Hebeloma*	Not reported		
Ascomycota	*Hyaloscypha*	Not reported		
Ascomycota	*Hypocrea*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Lecythophora*	Not reported		
Ascomycota	*Leptographium*	Not reported		
Ascomycota	*Meliniomyces*	Not reported		
Ascomycota	*Microcera*	Not reported		
Ascomycota	*Musicillium*	Not reported		
Ascomycota	*Mycocentrospora*	Not reported		
Ascomycota	*Mycosphaerella*	*Acropora hyacinthus*	*Arabian Sea*	Barathikannan et al. (2017)
Ascomycota	*Mycosphaerella*	Reported in brown algae	Not specified	Fries 1979
Ascomycota	*Ophiocordyceps*	Not reported		
Ascomycota	*Ophiostoma*	Not reported		
Ascomycota	*Pezizomycotina*	Not reported		
Ascomycota	*Phaeocollybia*	Not reported		
Ascomycota	*Phoma*	*Acropora formosa*	Great Barrier Reef	Yarden et al. 2007
Ascomycota	*Phoma*	*Leptogorgia* sp. *and L. obscura*	Ecuador	Soler-Hurtado et al. 2016
Ascomycota	*Phoma*	Not specified	Great Barrier Reef	Morrison-Gardiner 2002
Ascomycota	*Phoma*	Sponges	Singapore	Koh et al. (2000)

Phylum	Genus	Host	Location	Reference
Ascomycota	*Phoma*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Pichia*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Placomaronea*	Not reported		
Ascomycota	*Podospora*	Not reported		
Ascomycota	*Pseudaegerita*	Not reported		
Ascomycota	*Pseudeurotium*	Not reported		
Ascomycota	*Pyrenochaeta*	Reported in green algae	Not specified	Suryanarayana n et al. (2010)
Ascomycota	*Rhinocladiella*	Not reported		
Ascomycota	*Sarcinomyces*	Not reported		
Ascomycota	*Seimatosporium*	Not reported		
Ascomycota	*Sporopachydermia*	Not reported		
Ascomycota	*Stachybotrys*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Teratosphaeria*	Not reported		
Ascomycota	*Tetracladium*	Not reported		
Ascomycota	*Tetracladium*	Not reported		
Ascomycota	*Trichoderma*	*Gorgonia ventalina*	Puerto Rico	Toledo-Hernandez et al. 2008
Ascomycota	*Trichoderma*	Sponges	Singapore	Koh et al. (2000)
Ascomycota	*Trichoderma*	Artifical reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Trichoderma*	*Favia* sp.	Java, Indonesia	Cristianawati et al. 2017
Ascomycota	*Trichophyton*	Artificial reef biofilm	North-Central Gulf of Mexico	Salamone et al. 2016
Ascomycota	*Tuber*	Not reported		
Basidiomycota	*Antherospora*	Not reported		
Basidiomycota	*Auricularia*	Not reported		
Basidiomycota	*Derxomyces*	Not reported		
Basidiomycota	*Dictyonema*	Not reported		

Phylum	Genus	Host	Location	Reference
Basidiomycota	*Exidia*	Not reported		
Basidiomycota	*Exobasidium*	Not reported		
Basidiomycota	*Farysizyma*	Not reported		
Basidiomycota	*Fomitiporia*	Not reported		
Basidiomycota	*Frantisekia*	Not reported		
Basidiomycota	*Hyphodermella*	Not reported		
Basidiomycota	*Inocybe*	Not reported		
Basidiomycota	*Irpex*	Not reported		
Basidiomycota	*Mortierella*	Not reported		
Basidiomycota	*Mycena*	Not reported		
Basidiomycota	*Protomerulius*	Not reported		
Basidiomycota	*Psathyrella*	Not reported		
Basidiomycota	*Scleroderma*	Not reported		
Basidiomycota	*Thelephora*	Not reported		
Basidiomycota	*Tomentella*	Not reported		
Basidiomycota	*Trametes*	Not reported		
Basidiomycota	*Trichaptum*	Not reported		
Zygomycota	*Mycotypha*	Not reported		

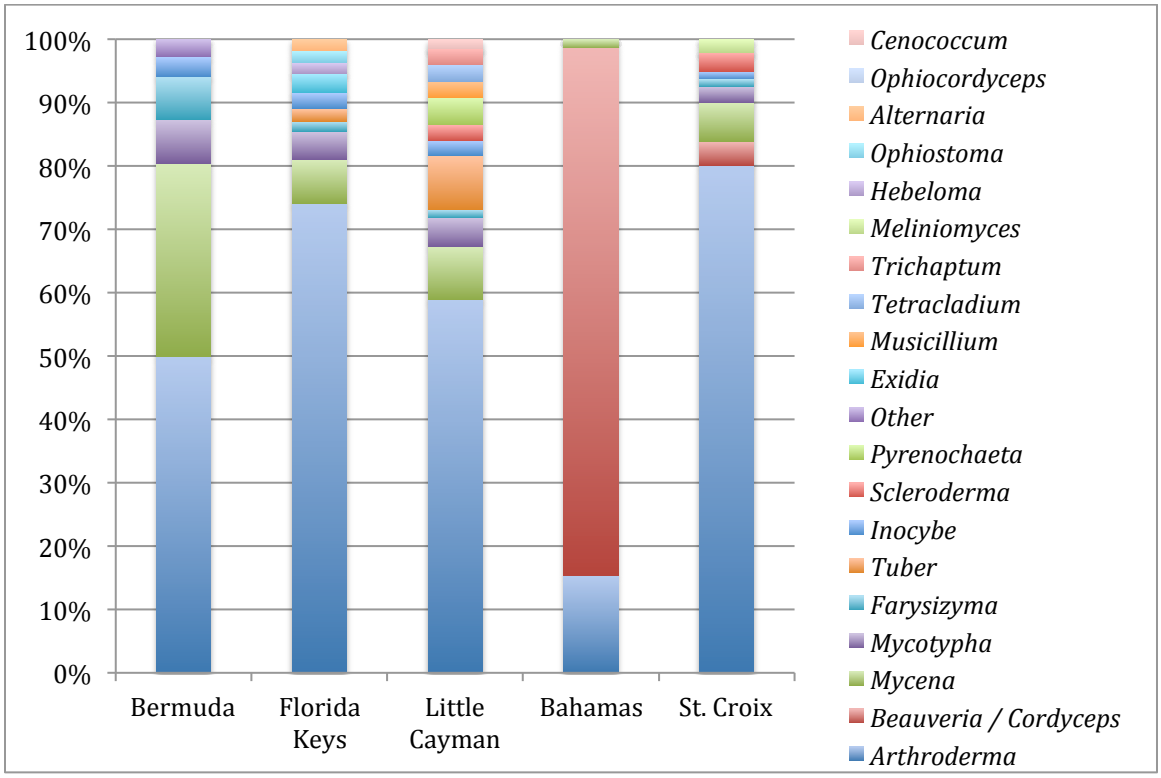

Figure 6. Histogram representing the averaged fungal community profile at each of the sampling locations. Samples from Bermuda are from *O. franksi;* samples from all other locations are from *O. faveolata.* Taxa are listed in order of their appearance in bars (from top to bottom).

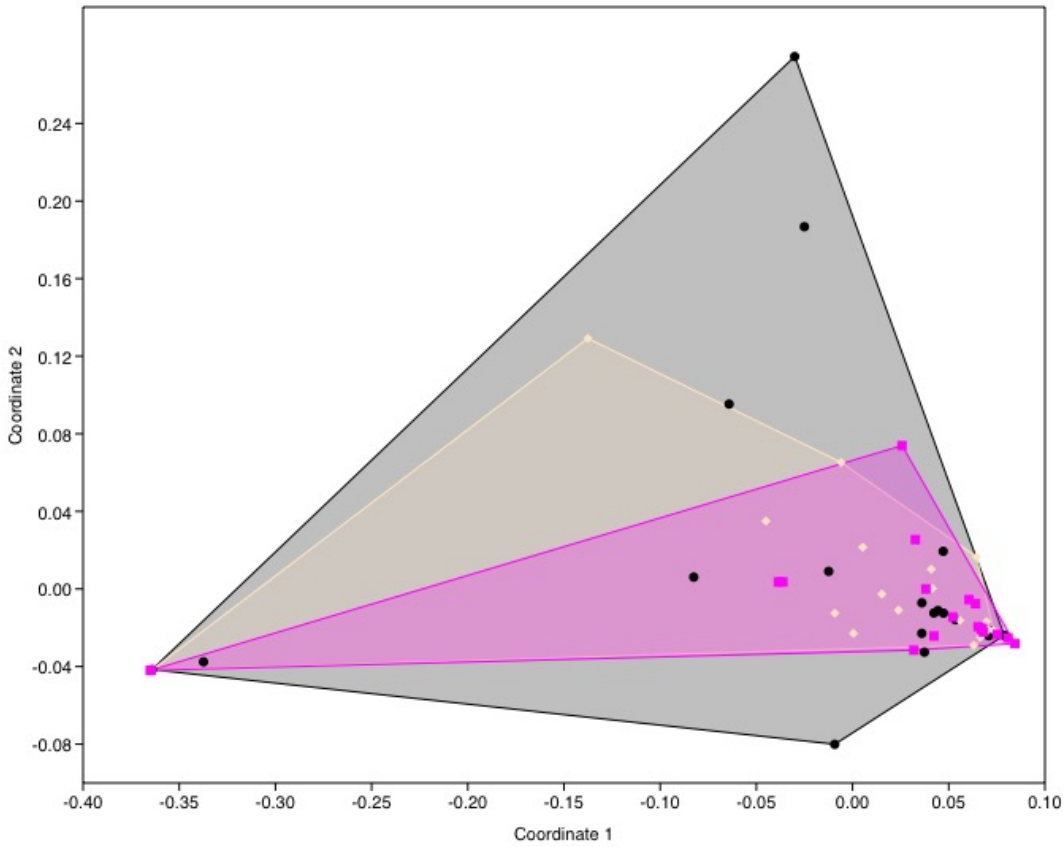

Figure 7. Two-dimensional scatter plot generated from NMDS, groupings of fungal communities were done according to health. Apparently healthy samples are represented by tan diamonds (◆) apparently healthy-on-diseased samples by pink squares(■) ,and diseased samples by black dots (•).

Table 3. Result of the PERMANOVA comparison of all fungal communities classified according to state of health. (p-value < 0.05)

	D	AH	AHD
D		0.974	0.8487
AH	0.974		0.7686
AHD	0.8487	0.7686	

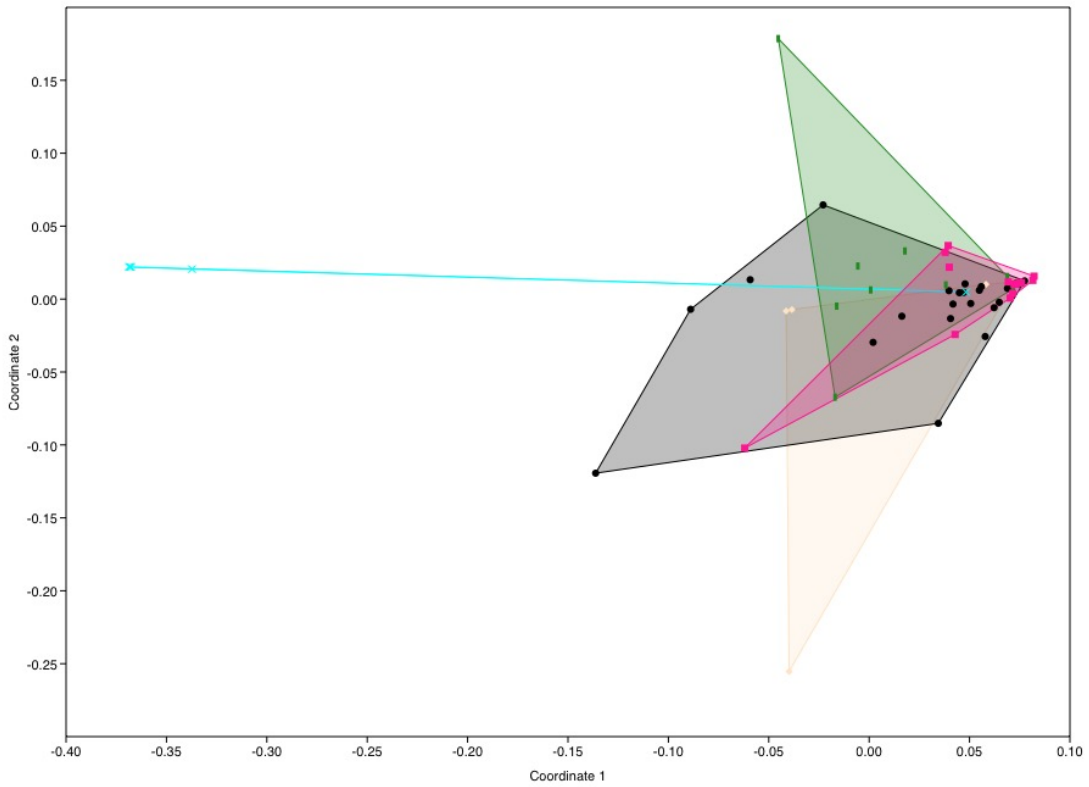

Figure 8. Two-dimensional scatter plot generated from NMDS, groupings of fungal communities were done according to location. Green bars (▮) represent Little Cayman Island, black dots (•) represent Florida Keys, tan diamonds (◆) represent Bermuda, pink squares (■) represent St. Croix, blue X's (X) represent Lee Stocking Island, Bahamas.

Table 4. Result of the PERMANOVA comparison of all fungal communities according to locations, showing that several groups (in red) were unique in composition (p-value < 0.05)

	BR	FKNMS	LC	LSI	STX
BR		0.022	0.1768	0.016	0.0094
FKNMS	0.022		0.1785	0.0002	0.0881
LC	0.1768	0.1785		0.0024	0.0061
LSI	0.016	0.0002	0.0024		0.0005
STX	0.0094	0.0881	0.0061	0.0005	

A local analysis exclusively using Florida Keys samples was conducted examining the communities of apparently healthy, diseased, and apparently healthy-on-diseased samples. Apparently healthy samples taken from the Florida Keys National Marine Sanctuary had an average richness of 11.9 taxa, apparently healthy tissue taken from diseased colonies had an average richness of 9.3 taxa and diseased samples had an average richness of 10 taxa. **(Table 5)**.

Results of a one-way ANOVA showed that the variations in taxa richness among health classes at the Florida Keys location were not statistically significant ($F=0.8278$) (d.f.=2) ($p=0.45$). Following indicator species analysis, no indicator values were shown to be statistically significant.

Within the Florida Keys site, the dominant fungal genus regardless of health was *Arthroderma*. However, this genus occupied a larger percentage of the total community in diseased and apparently healthy-on-diseased colony samples compared to healthy samples. Other quantitative differences between these samples included *Exidia*, being more abundant in apparently healthy samples, while larger percentages of the community were comprised of *Inocybe*, *Mycena*, and *Arthroderma* in apparently healthy-on-diseased and diseased samples **(Figure 9)**.

Table 5. Fungal taxa richness of samples taken from the Florida Keys National Marine Sanctuary classified according to health.

Fungal Richness	Average Taxa
Apparently Healthy	11.9
Apparently Healthy on Diseased	9.3
Diseased	10

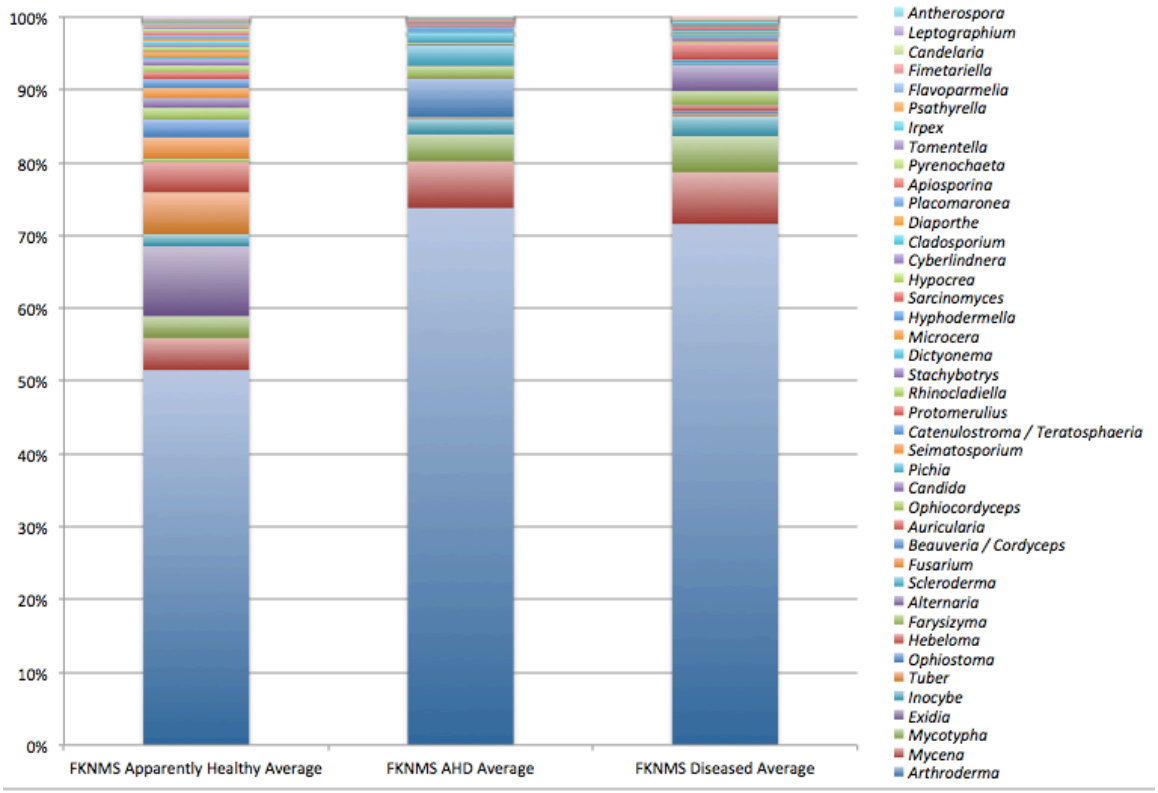

Figure 9. Histogram representing the averaged fungal community profile of the Florida Keys (FKNMS) samples classified according to disease state.

Non-metric multidimensional scaling was applied to the fungi identified from the Florida Keys samples to generate an ordination plot with respect to health **(Figure 10).** Pairwise comparisons within this location showed that fungal communities of apparently healthy samples were distinctive from both diseased and apparently healthy-on-diseased samples **(Table 6)**.

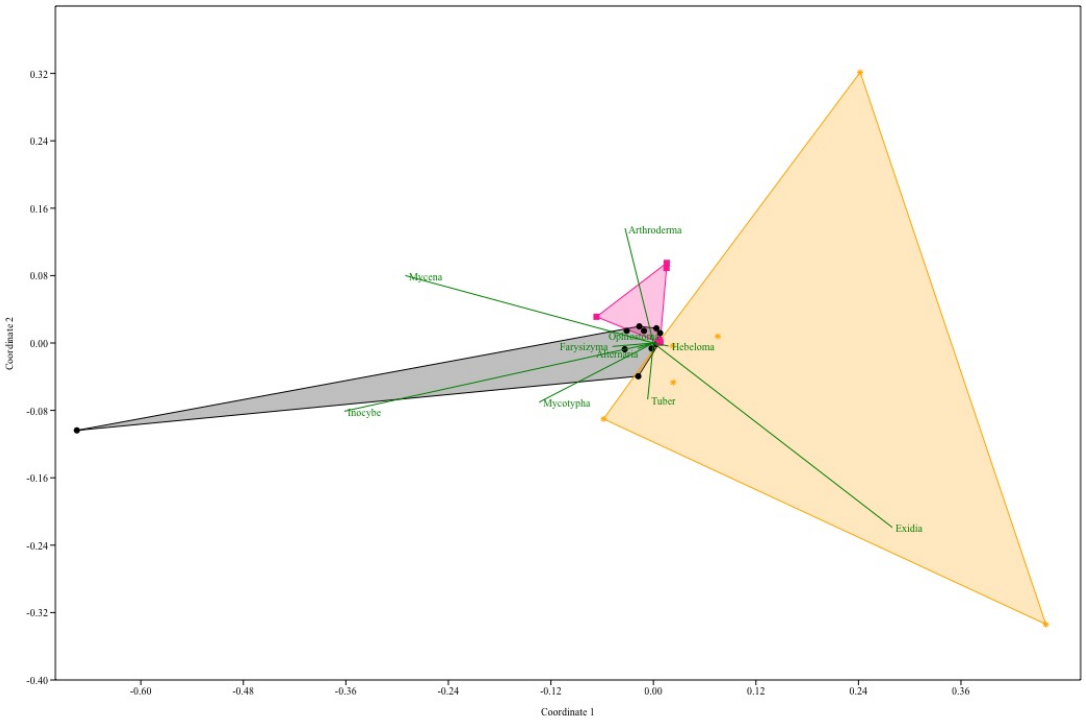

Figure 10. Scatter plot displaying ordination of fungal communities by coral health according to non-metric multidimensional scaling, from the samples taken in the Florida Keys National Marine Sanctuary. Healthy samples are represented by tan diamonds (◆) healthy-on-diseased samples by pink squares(■) and diseased samples by black dots (•).

Table 6. Result of the PERMANOVA comparison, showing that fungal communities in healthy (AH) samples were distinct (p-value < 0.05 in red) from both healthy-on-diseased (AHD) samples and diseased (D) samples.

	D	AH	AHD
D		0.0473	0.9216
AH	0.0473		0.0423
AHD	0.9216	0.0423	

Discussion

Isolation of Fungal DNA from Coral DNA

The coral holobiont is not only comprised of prokaryotic archaea and bacteria but also eukaryotic members such as algae, sponges, worms and fungi. DNA amplification and sequencing techniques have proved successful in effectively amplifying and detecting target organisms from multiple kingdoms. The focus of this study was the fungal community yet trying to amplify DNA exclusively from this kingdom proved difficult.

Samples of *Orbicella* were initially taken for bacterial community assessment (Cook 2009). Subsequently, those samples also were subjected to further analysis regarding the fungal community composition. Traditionally, a variable region of the 16S rRNA gene is amplified and sequenced for bacterial identification. Fungal taxonomic and phylogenetic studies focus on comparing sequences of the ITS region in fungi. To identify the fungi within the coral host, this region was amplified and sequenced using recommended fungal primers (White et al. 1990; Moore et al. 2011). Despite many attempts, targeting the fungal ITS1 region from a coral sample was problematic because the fungal ITS primers also amplified the much more abundant coral DNA. Therefore, DNA from coral samples was amplified, sequenced,

and then fungal sequences identified, with all other sequences eliminated from further analysis.

The methodology utilized in this study included two taxonomic databases: NCBI nucleotide BLAST® and RDP 11 database. The RDP 11 database does not have taxonomic information for animals, only bacteria, archaea, and fungi. Therefore, if an animal sequence is submitted, RDP 11 will assign a fungal taxonomic identification, coral being more closely related to fungi than bacteria or archaea (Cole et al. 2014). This is a significant problem since coral DNA is far more abundant in the coral samples compared to the fungal DNA (Ravindran et al. 2001; Littman et al. 2011; Garcia et al. 2013). Therefore, all non-fungal sequences were eliminated based on the BLAST taxonomic designation prior to submission to RDP 11. In this way, fungal sequences were assigned the correct taxonomic designation using the much more accurate and faster RDP classifier (Cole et al. 2014). All 799,294 PGM generated sequences were subjected to a nucleotide BLAST® search through George Mason University's MBAC Galaxy Portal. Following taxonomic assignment by sequence, all non-fungal sequences were manually eliminated from the FASTA file before further analysis. The remaining 130,177 fungal sequences were identified using the RDP 11 database.

Separating fungal DNA from the coral host has been attempted in a few instances, with noted difficulties. Barathikannan et al. (2017) isolated fungi from coral mucus by culturing fungi on selective growth media to restrict bacterial growth. Fungal colonies were then transferred to different agar plates containing

sterile coral mucus (Barathikannan et al. 2017). Molecular identification of the isolates was done using ITS1 and ITS4 primers (Barathikannan et al. 2017). Similarly, Domart-Coulon et al. employed a culture-dependent approach as well, isolating a strain of *Cryptococcus* sp. (2004). Ravindran et al. (2001) estimated the fungal biomass of the holobiont to be 0.05% of total weight. Garcia et al. (2013) similarly noted the low abundance of fungal DNA in coral tissue, reporting only 131 fungal sequences within a total 65,085 sequences (0.002%). The methodology presented here (i.e., post-sequence exclusion of non-pertinent DNA sequences) offers an effective and convenient solution to the problem of identifying target sequences when primers amplify both the desired and undesired DNA.

Histology

Histological preparations served a two-fold role for this study: positive identification of the correct coral species and documentation of the fungi within specific parts of the coral. *Orbicella annularis*, *O. faveolata*, and *O. franksi* have been shown to be distinct species, but field identification can be difficult due to the many shared morphologies between the species. By histologically confirming the samples were in fact *O. faveolata* and not a congener served as a quality control. H&E-stained samples of *O. faveolata* were inspected for the presence of specific eosinophilic cells, characteristic of this species, but lacking in *O. franksi* (personal communication with E. Peters) **(Figure 11)**.

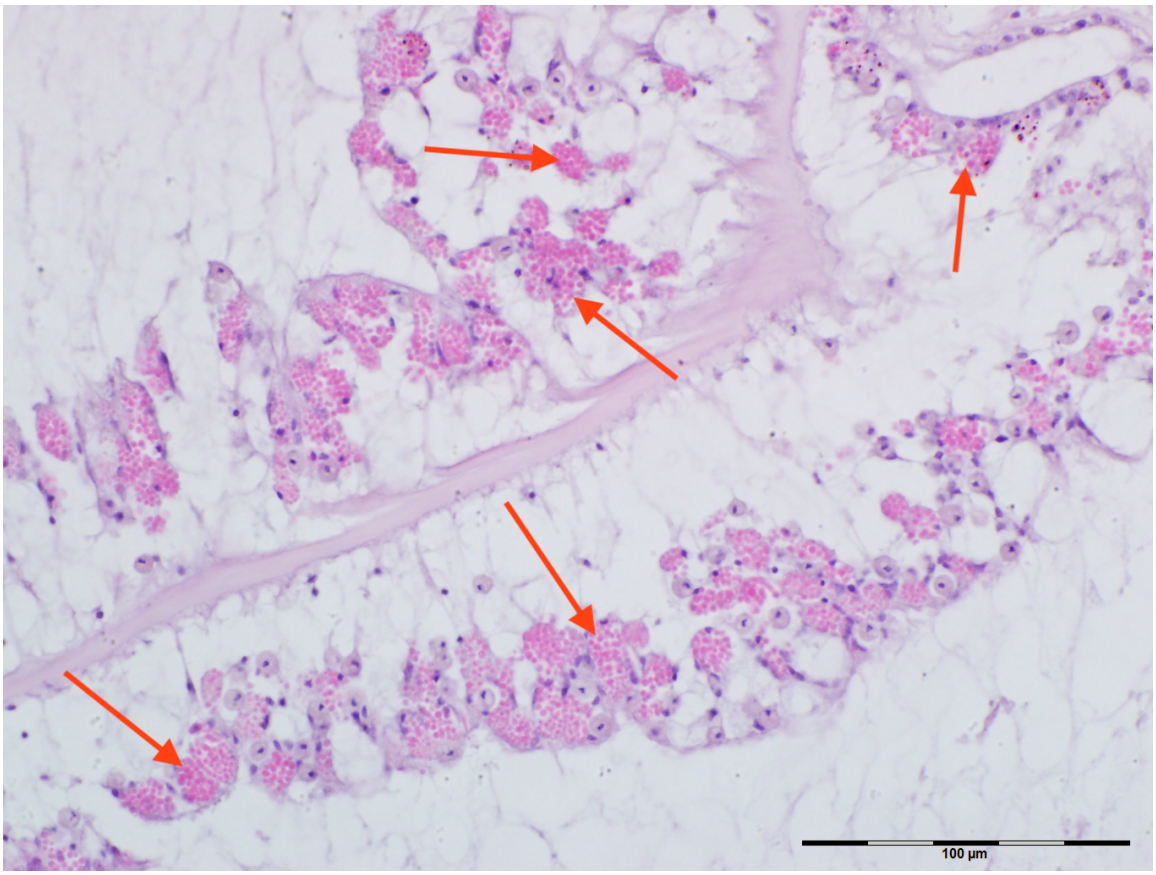

Figure 11. H&E 400x magnification, Eosinophilic cells (red arrows) attached to the mesentery of *O. faveolata*. These cells are characteristic of this particular species allowing for confirmation that the correct species was obtained.

Positive preferential staining, along with characteristic morphology confirmed a fungal presence within coral samples. Uniform diameter of the endolithic filaments, along with the positive histological staining (AB/PAS/H) helped to confirm the presence of fungi within the skeleton **(Figure 12).** Positively stained structures were consistent with that of fungal growth patterns previously reported (Kendrick et al. 1982; Le Campion-Alsumard et al 1995a; Le Campion-Alsumard et al 1995b; Bentis et al. 2000; Priess et al. 2000; Golubic et al. 2005; Renegar et al. 2008). In some instances, much larger septate hyphae were observed **(Figure 13)**. Larger hyphae are assumed to be cryptoendoliths (Priess et al. 2000; Domart-Coulon et al. 2004), and finer hyphae are assumed to be euendoliths (Priess et al. 2000; Domart-Coulon et al. 2004). Nearly all preferentially stained (AB/PAS/H) slides had coloration indicative of fungi (purple or magenta) and presented cells shaped as hyphae within the skeleton.

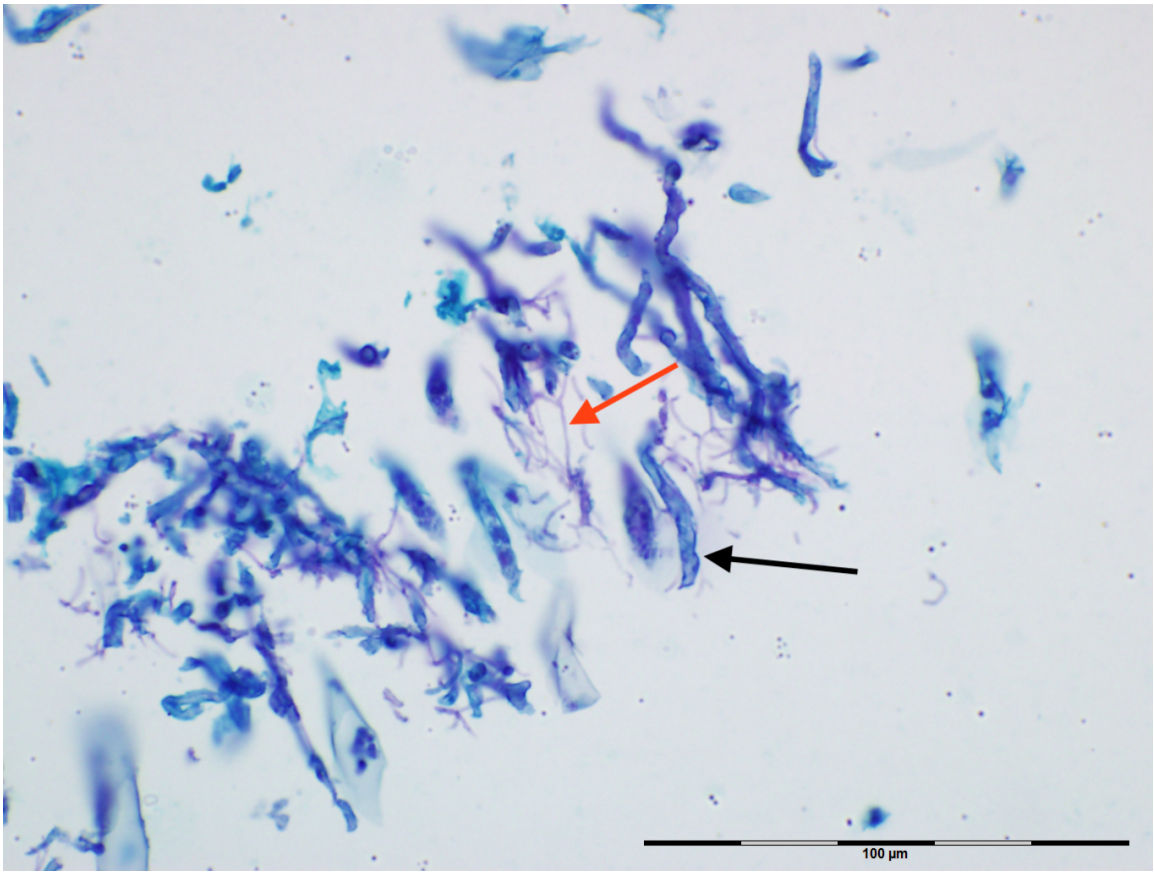

Figure 12. AB/PAS/H 400x magnification, Endolithic organisms found within the skeleton of a seemingly healthy sample of *O. faveolata*. Note the size difference between fungal hyphae (red arrow) and endolithic algae (black arrow).

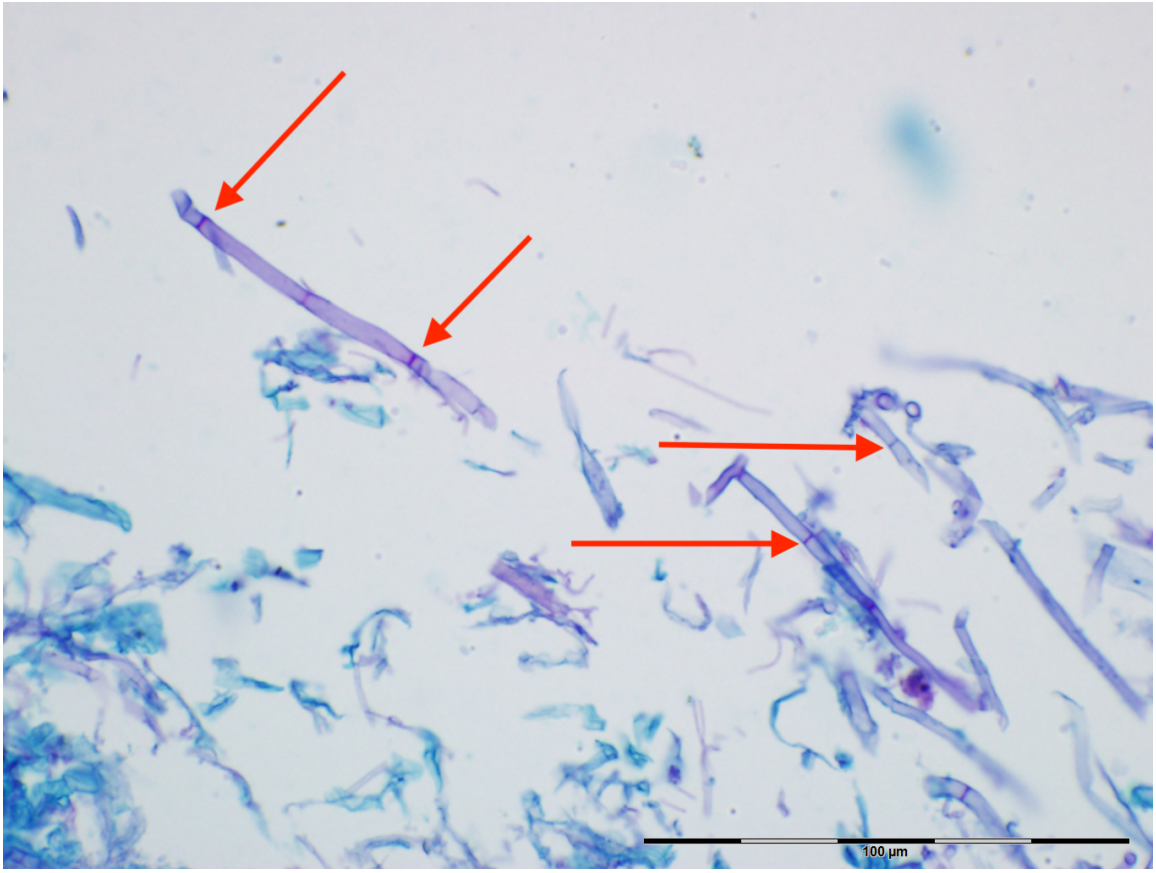

Figure 13. AB/PAS/H 600x magnification, Endolithic community found within an apparently healthy sample of *O. faveolata*. Note positively stained fungi that are displaying what appear to be septa (red arrows).

Endolithic organisms observed in this research were separated from the coral basal body wall, with the exception of two infections where endolithic fungi were also penetrating the body wall **(Figures 3** and **4).** Endolithic fungi burrow from the skeleton interior upward at a pace that equals the rate of coral aragonite deposition (Le Campion-Alsumard et al 1995a; Le Campion-Alsumard et al 1995b; Golubic et al. 2005). Under conditions considered typical, coral tissue is reported to avoid direct contact with the fungi by maintaining a consistent pace; most of the histological samples produced in this study also displayed this **(Figure 14).**

Even with thorough scrutiny, no reproductive structures were observed. This apparent absence is not unprecedented among studies that include histological methods; only a few have noted conidiophores (for positive confirmation see: Bak and Laane 1987; Priess et al. 2000). The coral skeleton presents an alien environment for many terrestrial-based fungi, which typically depend on aerial dispersal of spores and specific environmental cues for reproduction (Gleason et al. 2017b). Several sources note the presence or potential presence of fungi in other locations of a coral polyp (Raghukumar and Ravindran 2012; Damare et al. 2012; Salamone et al. 2016) but identifying them histologically proved unsuccessful in this study.

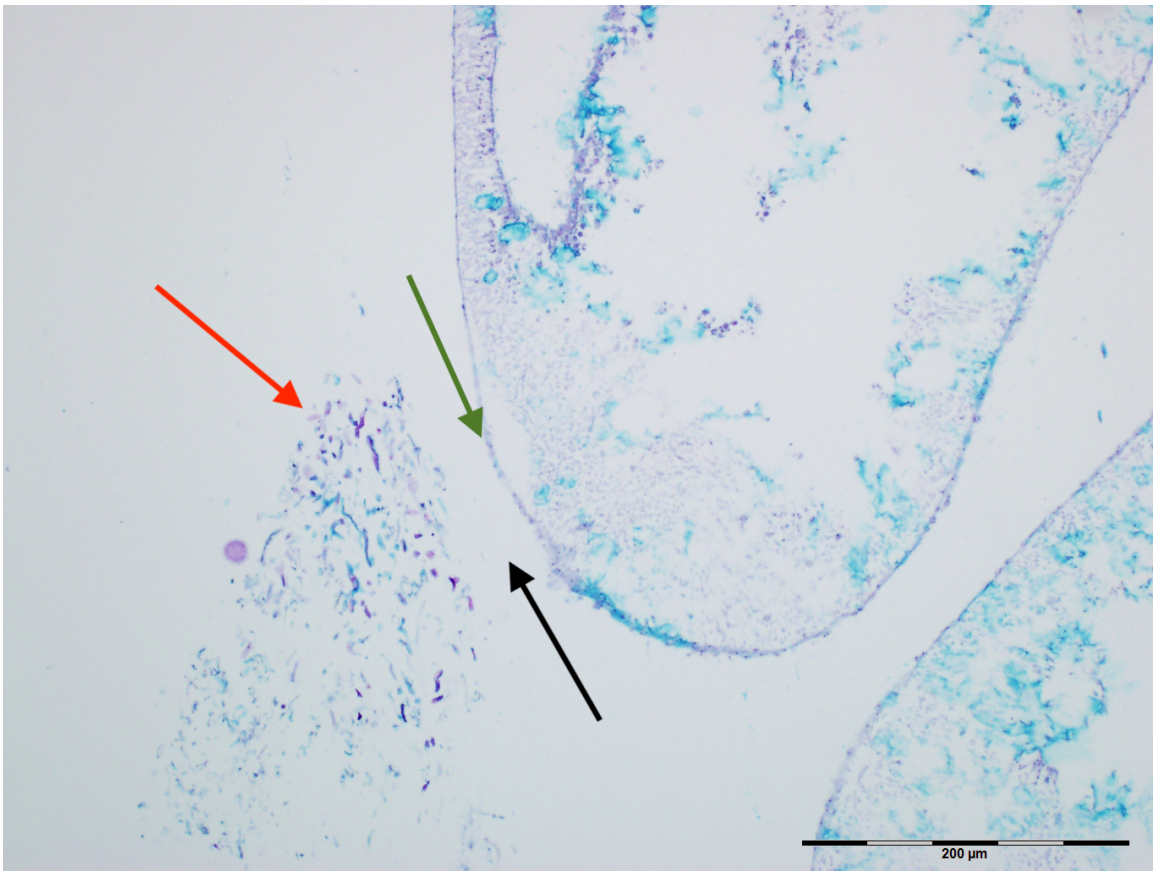

Figure 14. AB/PAS/H 100x magnification, Endolithic community found within *O. faveolata* (red arrow). Note the space (black arrow) between the basal body wall (green arrow) and the endolithic community

Fungal Diversity Within the Coral

Many studies have noted a more abundant fungal presence in diseased coral specimens (Bak and Laane 1987; Raghukumar and Raghukumar 1991; Thurber et al. 2009; Littman et al. 2011). This was not observed in the present study. Among all locations, or when exclusively examining the Florida Keys location, fungal richness was highest in apparently healthy samples **(Tables 1** and **5).** However**,** one-way ANOVA showed that taxa richness among all samples was not significantly different. These results refute the second hypothesis, $\mathbf{HA_2}$, that greater fungal richness would be observed in samples of *Orbicella faveolata* exhibiting signs of white plague-like disease.

The findings here were shared by Garcia et al. (2013) who observed a similar phenomenon, reporting a richness among 12 total samples (six apparently healthy and six diseased) that contained 108 metagenomic sequences from Ascomycota fungi and 23 from Basidiomycota fungi. No statistical difference between the diversity of healthy and affected coral was found.

Furthermore, the data here show that there were no fungi specifically indicative of diseased or non-diseased samples (Figure 5). The Indicator species analysis confirmed that none of the fungal taxa were individually responsible for the observed differences between coral health groups. These results refuted the third hypothesis, $\mathbf{H3_A}$, that specific fungi would be present in diseased samples.

Fungal Communities Cluster By Location

A one-way PERMANOVA pairwise comparison of fungal communities from corals in different locations partially supported the first alternative hypothesis, **H1$_A$**, that fungal communities varied based on geographic location. Both family and genus level analyses confirmed the geographic variations (**Figure 8** and **Table 4**). Geographic location may not be the only deterministic factor in fungal community variation. For instance, the coral fungal communities from the Cayman Island sampling location were not found to be significantly different from those at the Bermuda location despite the long distance between the two sites. Therefore, other influencing factors may be involved, such as proximity to shore (Morrison-Gardiner 2002), nutrient profile of the water (Kim and Harvell 2004 Patterson et al. 2002; Klause et al. 2007), primary production (Gutiérrez et al. 2011), turbidity (Kuta and Richardson 2002; Richardson and Kuta 2003), day vs. night fluctuations (Moree et al. 2014), seasonal fluctuations (Gutiérez et al. 2011), age of the coral colony (Williams et al. 2015), salinity (Taylor and Cunliffe 2016) and temperature (Alker et al. 2001; Kuta and Richardson 2002; Patterson et al. 2002). Given the above, you would expect the Lee Stocking Island fungal communities to be the same as those at Little Cayman, but they are not. Again, other factors listed above could explain the differences.

Results in the current study showed that location in some cases influenced fungal community composition (**Figure 8** and **Table 4**). The effects of location, however, appeared to mask any effect of health status (AH vs. D vs. AHD) when all locations were considered together. Therefore, the effect of health status on fungal

communities was examined in just one location, the Florida Keys National Marine Sanctuary.

Florida Keys Samples Cluster by Health

Samples taken from a single location (Florida Keys National Marine Sanctuary) showed that fungal communities did differ by health status **(Figure 10** and **Table 6)** when a single location was examined. This refutes the second null hypothesis $\mathbf{H2_0}$ that fungal communities would not show variation by health. This location was the only region with a sufficiently sized and evenly grouped set of samples to conduct statistical analysis. The fungal communities from apparently healthy samples were statistically distinct from diseased and apparently healthy-on-diseased samples as shown by a PERMANOVA one-way pairwise comparison (p-value <0.05; **Table 6**), whereas diseased and apparently healthy-on-diseased samples showed overlap.

Analysis of the FKNMS profile **(Figure 9)** showed an apparently larger percentage of the community to be composed of. *Arthroderma* in apparently healthy-on-diseased and diseased samples compared to apparently healthy samples at this site. Nonetheless, *Arthroderma* sp, was not found to be specifically associated with diseased tissues (i.e., an indicator species) and the profiles for the different health states were similar in terms of taxa present. Therefore, the difference between apparently healthy tissues and diseased tissues as indicated by the NMDS ordination may be due to differences in the overall distribution or relative

abundances of fungi rather than due to the presence of any specific fungus or group of fungi. For example, apparent changes in the abundances of *Arthroderma,* "*Exidia***,** *Inocybe, and Mycena* among the different health states **(Figure 9)** could reflect changes in the health status of *O. faveolata.* This would be consistent with the hypothesis that changes in the mycobiome of healthy tissues can result in disease, such as when opportunistic fungi become pathogenic when the host is stressed (Weil 2004; Barrero-Canosa et al.,2013; Yarden, 2014).

Most of the conclusive studies concerning WPLD implicate bacteria (Richardson et al. 1998a; Richardson et al. 1998b; Richardson et al. 2001; Denner et al. 2003; Thompson et al. 2006; Bourne et al. 2009; Ainsworth et al. 2017). However, there are known fungal pathogens of coral, including *A. sydowii*, which has been shown to infect the soft corals *Gorgonia flabellum* and *G. ventalina* (Smith et al. 1996; Alker et al. 2001; Toledo-Hernández et al. 2008), *Leptogorgia* sp. and *L. obscura* (Soler-Hurtado et al. 2016). Several other studies have suggested a fungal role in coral diseases (Le Campion-Alsumard et al. 1995a; Bentis et al. 2000; Priess et al. 2000; Ravindran et al. 2001; Raghukumar and Raghukumar 1991; McClanahan et al. 2004; Yarden et al. 2007; Renegar et al. 2008; Work et al. 2008).

Renegar et al. (2008) compared tissue and skeletal samples of *Siderastrea siderea* and *Agaricia agaricites* from Lee Stocking Island and Little Cayman. Histological and structural comparison of dark spot syndrome (DSS) and apparently healthy samples showed endolithic fungal hyphae near the calicodermis in diseased *S. siderea,* but not in apparently healthy samples. The authors, however, could not

find fungal hyphae in DSS-affected *A. agaricites* samples taken from the same locations (Renegar et al. 2008). Because the two species of coral were taken from the same location and exhibited similar phenotypic signs of infection, this presence and absence of fungi between the two species led the authors to speculate, "that the causal agent of DSS in these two coral species may be different" (Renegar et al. 2008).

Similarly, the "one disease-one pathogen" paradigm may not always be true since *A. sydowii* is not always present in diseased *Gorgonian* species (Toledo-Hernández et al. 2008) and, conversely, *A. sydowii* can be found in apparently healthy sea fans (Soler-Hurtado et al. 2016; Gleason et al. 2017b). Perhaps there is more than one potential pathogen for the same set of diseases (Weil 2004; Renegar et al. 2008; Sunagawa et al. 2009; Work et al. 2012; Kellogg et al. 2013; Roder et al. 2014; Work and Meteyer 2014). This paradox, led Gleason et al. (2017b) to conclude, "Since *A. sydowii* can be present in healthy corals, perhaps environmental factors are involved as well," a hypothesis also proposed by Weil (2004), Barrero-Canosa et al. (2013), and Yarden (2014).

Conclusions

Orbicella faveolata is a complex animal hosting a diverse collection of organisms, including fungi. Suspected to play roles in certain nutrient cycles and as potential pathogens, the contributions and impacts fungi have on their hosts are nevertheless insufficiently understood. Corals are limited in phenotypic expression, showing only a narrow set of responses, even when affected by diverse pathogens. In this way, some responses may seem similar despite having different causes. Coral samples from colonies appearing to show signs of white plague-like disease were compared with apparently health coral samples.

Evidence found in this study suggests that the effect of location on fungal community composition can mask underlying correlations between fungal communities and coral health status. The effect of health status was revealed when observations were limited to one location, the Florida Keys National Marine Sanctuary. Here, apparently healthy samples contained statistically distinct fungal communities compared to diseased and apparently healthy-on-diseased colonies. Despite this correlation, no specific fungus was observed to be associated with either the apparently healthy or diseased state of the coral; only changes in overall relative abundances of the fungal genera were detected. This suggests that a shift in community composition *per se* may be more important than the presence of any one

fungus or group of fungi.

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